

Development of a Vaccine for
Bacterial Kidney Disease in Salmon

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by

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EXECUTIVE SUMMARY

Bacterial kidney disease (BKD) has been and remains a chronic contributory problem limiting the productivity of salmon in the Columbia River Basin. Control of this disease will not come easily, but it would lead to a tremendous increase in the health and numbers of our salmon populations. Vaccination of salmon to Renibacterium salmoninarum (KDB) is a potentially successful method of controlling this disease. To date, however, no successful vaccine has been developed for general use. A possible solution to this problem, and thus the goal of this research, is to isolate the antigenic components of KDB and enhance their ability to activate the host defenses. This will be accomplished by the chemical modification of these antigens with potent immunomodulatory substances. These modified antigens will then be tested for their effectiveness in inducing immunity to BKD and thereby preventing the disease.

The goal of the project's fourth year was to test the immunogenicity and prophylactic value in coho salmon (Oncorhynchus kisutch) of various chemical conjugates of Renibacterium salmoninarum cell and major antigens. This was accomplished by assessing the serum antibody response, the cellular immune response (chemiluminescence), and the kinetics of mortality after lethal injections of the bacteria.

The studies completed this year have: 1) identified immunization procedures which enhance the induction of high levels of antibody; 2) identified functionally distinct serum antibodies which may possess different abilities to protect salmon against BKD; 3) begun the isolation and characterization of anti-R. salmoninarum antibodies which may correlate with

varying degrees of protection; 4) identified chemiluminescence as a potential method for assessing cellular immunity to bacterial kidney disease; and 5) characterized two monoclonal antibodies to R. salmoninarum which will be of benefit in the diagnosis of **this** disease.

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INTRODUCTION

Geographical Distribution

Bacterial kidney disease (BKD) is one of the most widespread diseases in the Columbia River Basin and occurs in many parts of the United States, Canada, Europe (Fryer and Sanders, 1981) and Japan (Kimura and Awakura, 1977). The first reported identification of what was most likely R. salmoninarum or kidney disease bacterium (KDB), was in 1930.

Smith (1964) communicates that gram positive diplococci were isolated from Atlantic salmon (*Salmo salar*) found in Aberdeenshire in the River Spey in Scotland. The first recorded cases in the United States occurred in hatcheries in the state of Massachusetts as described by Belding and Merrill (1935). Not only is this disease a very grave problem for hatchery reared salmonids, but it has also been demonstrated to occur in wild populations (Evelyn, et al., 1973).

The Pathogen

The kidney disease bacterium (RDB) is described as a non-motile, non-spore forming diplobacillus. Original attempts to isolate the organism failed due to its rather fastidious requirements for growth in culture media. The first successful attempts at cultivation utilized media with such rich supplements as beef serum and fish extract (Earp et al., 1953). Later work of Ordal and Earp (1956) made use of cysteine blood agar, which contained 20% human blood as well as 0.1% cysteine-HCl. The most recent modifications obviates the need for blood

or serum thus reducing the cost. This medium employs 0.1% L-cysteine-HCl in Mueller Hinton medium (Wolf and Dunbar, 1954).

Superficially the organisms, especially when seen within the tissues, resemble corynebacteria, thus they have been referred to as corynebacteria and the disease as corynebacterial kidney disease (Ordal and Earp, 1956; Hunn, 1964; Wedemeyer and Ross, 1973). Certain aspects of the pathology caused by this organism resemble not only corynebacterial disease, but to some degree diseases caused by mycobacteria and listeria. However, these rather circumstantial methods of characterization were not satisfactory for rigorous taxonomic classification. Fryer and Sanders (1981) explored this taxonomic problem on the molecular level, and determined that in regards to the guanosine/cytosine (GC) content, peptidoglycan and cell wall composition, these organisms were quite unique. Due to these singular molecular characteristics, these organisms have been placed in their own genus and species, Renibacterium salmoninarum (Fryer and Sanders, 1980). Isolates from various regions of the world also seem to share only a single serotype (Getchell, 1985).

Disease Pathology

The disease caused by RDB is considered to be a chronic systemic disease, with lesions occurring through much of the viscera and musculature in advanced cases. A common route of entry for the organism has not been demonstrated, and it seems plausible that infection may occur by various routes. Wood and Wallis (1955) have demonstrated that the ingestion of infected salmon flesh by chinook salmon leads to a lethal infection. Alternatively, eye trauma has also been suggested as a possible route of infection. Hendricks and

Leek (1975) found that chinook salmon possessing exophthalmia, demonstrated granulomatous lesions behind the affected eye which contained large numbers of leukocytes and KDB. Many of the fish which possessed this exophthalmia, had no other lesions internally. However, if all fish which exhibited this exophthalmia were held in aquaria, it was found that they all succumbed to BKD within two to three months. Upon necropsy, the animals were found to have disseminated BKD lesions. It was felt that the eye may serve a primary route of infection in hatchery-reared salmonids, due to the frequency of eye trauma when the animals are maintained in raceways. A similar, but perhaps a more common route of infection, may be through general abrasion of the body surface (Wolf and Dunbar, 1959).

Although there are many organs which become infiltrated with KDB, most investigators feel that the primary target is the kidney. The hematopoietic portion of the anterior kidney appears to be especially susceptible. It is a diagnostic feature of the disease to see white granulomatous areas of infection within the kidney. When examined microscopically these areas are seen to possess KD organisms. These foci of infection are not limited to the kidney, but they are found to appear also in the spleen and liver. As the disease progresses, the reproductive organs, musculature, and brain often become infected. Externally, besides the exophthalmia, pustules or blebs may be seen above the lateral line and petechial hemorrhaging around the muscles of the peritoneum.

One of the most striking features of the internal pathology of BKD is the development of a white pseudomembrane composed of dead host tissue, bacteria, and leukocytes. This false membrane has been seen to cover the liver, reproductive organs, spleen, and

occasionally the swim bladder (Snieszko and Griffin, 1955). This phenomenon is **not** unlike the pseudomembrane produced in diphtheria infections in man. The formation of this pseudomembrane, however, is **quite** temperature dependent. It is reported that it forms at temperatures below 8.3°C, whereas at higher temperatures only necrosis is found (Smith, 1964). Attempts to isolate an exotoxin as is expressed by Corynebacteria diphtheriae have not met with success.

On the cellular level, bacterial kidney disease appears to be more similar to listerial or mycobacterial infections. A common feature of these diseases are the granulomatous reactions that occur. Like listeria and mycobacteria, KDB organisms are phagocytized by macrophages, but are not always digested by the phagocytic cells (Young and Chapman, 1978). In fact, the KDB as well as listeria and mycobacteria have been observed to multiply within the macrophage itself. In the case of mycobacteria, the cellular arm of the immune response is eventually activated **to** destroy the bacteria. The usual delay in this response, coupled with physiological mechanisms the pathogen uses to subvert the phagocytic response leads to a widespread infection. With regards to a specific antibody response, intracellular organisms such as KDB are thought to be relatively resistant, while within their host cell. In this situation, although the pathogen may be protected, the immune system of the host is still exposed to a continuous supply of antigen from the pathogens. As a result there is a continuous severe immune reaction which eventually destroys the surrounding host tissue in attempting to destroy the pathogen. This immune reaction leads to much necrosis and characteristically severe granulomatous lesions. Since KDB is harbored by macrophages of the fish and these severe granulomatous reactions occur in response to the antigen of the pathogen, it is not surprising that two of the organs that are most severely affected are the spleen and kidney. Both

of these organs are immune organs and contain a great number of **macrophages**.

Effect on the Kidney

It is felt that terminal cases of BKD may be fatal due to the destruction of the kidney which, in turn, may lead to an inability of the salmon to osmoregulate (Frantsi et al., 1975). This feature of BKD is extremely important in light of the evidence that salmon infected with BKD demonstrate marked increase in mortality when held in salt water as compared being held in fresh water (Banner et al., 1983). The possibility arises that even if fish seem relatively **healthy** or have recovered from BKD after antib-iotic treatment, they may be at high risk once they enter the ocean, due to extensive kidney damage.

Kidney pathology, in salmon **with** BKD, looks quite similar to the pathology seen in glomerulonephritis in mammals. Glomerulonephritis could be mediated by either of two mechanisms: 1) immune complex formation between KDB antigens and anti-KDB antibodies or, 2) by a reaction of anti KDB with crossreactive kidney antigen on the basement membrane of the glomerulus, The precise mechanism by which this condition is elicited has yet to be discovered, however, due to the degree of bacterial infiltration that occurs throughout the body of the fish, it would seem most likely that an immune complex reaction could be occurring.

Tolerance

It seems obvious that in most cases of BKD the salmon are responding to the pathogen, but their response seems inappropriate and incapable of overcoming the pathogenic insult. Immunological tolerance may

play a role in BKD if it serves to delay or produce an inappropriate immune response.

If a foreign antigen is present within the body early in life, the animal may experience a state of immunological tolerance (Billingham et al., 1953). In this state the animal does not recognize that particular foreign antigen as being different from its own body and; therefore, it will not respond to it immunologically. This phenomenon may apply in some cases to BRD. It **has** been demonstrated by Evelyn et al. (1984) that eggs from females with BKD possess the pathogen within the yolk. This was demonstrated microscopically and by iodine treatment of the egg surface. The iodine treatment should have killed any pathogen on the surface of the egg or residing within the perivitelline space, but the organism persisted. If this evidence is correct, it would support the hypothesis of vertical transmission of BKD and would indicate a route by which fry might experience conditions similar to that giving rise to neonatal tolerance in mammals.

Induction of Protective Immunity

The appearance of circulating antibody does not correlate with protection from the disease. Although this intracellular location of the pathogen may seem inaccessible, it is not. Mycobacteria and listeria also reside within phagocytes and these pathogens can be controlled if the host is properly sensitized or immunized. Listeria, which possesses physical characteristics similar to those of KDB (Bullock et al., 1975), appears to be insensitive to specific antibody. Priming of the cell mediated immune response, however, results in the elimination of these pathogens. Induction of the cell mediated (T cell) response results in the activation of the phagocytic cell and digestion of the bacteria residing there (Mackaness, 1969).

It is possible to enhance this cell mediated immune response, as well. as the immune response in general through the use of adjuvants. Adjuvants utilizing bacteria such as mycobacteria or corynebacteria lead to an enhancement of the immune response to the admixed antigen. Such augmentation may aid in the control of BKD. It has been reported that intraperitoneal injections of KDB emulsified in oil. and mycobacteria (Freund's complete adjuvant) can lead to a reduction of BKD lesions and organisms (Paterson et al., 1981). Although such immunization procedures would be impractical for large scale vaccine programs, they do demonstrate that proper presentation of KDB antigen to the fish can lead to a protective state of immunity.

MATERIALS AND METHODS

Animals.

Fingerling coho salmon (30-70 g) were kept in ambient (12°C) pathogen free well water, in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL). The fish were maintained on Oregon Moist Pellets. The disease challenges were performed on salmon housed at the OSU-FDL in 30-liter tanks, the effluent from which was chlorinated.

Adult female New Zealand White (NZW) rabbits and BALR/c mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strains.

Renibacterium salmoninarum isolates were a gift from Dr. J.S. Rohovec and C. Banner, Dept. of Microbiology, Oregon State University. The origins of the 10 isolates are listed in Table I. In addition, five control bacteria were also used: Streptococcus facium, S. fecalis, Lactobacillus piscicola, Vibrio anguillarum and Aeromonas salmonicida.

Growth conditions.

Bacteria were grown in either unfiltered Kidney Disease Medium-2 (KDN-2), ultrafiltered KDM-2 (UF-KDM-2), or RDM-II with 10% calf serum (Gibco, N.Y.) as originally specified by Evelyn (1977). The UF-KIN-2 was prepared by passage of KDM-2 through a PTGC-10,000 NMWL filter packet in a Manitan ultrafiltration apparatus (Millipore Corp., Bedford, MA). This filtration produced media free of molecules with molecular weights greater than 10,000. Cultures were incubated for one to three weeks in low form culture flasks with constant agitation at 17°C. At the end of the incubation period, the bacterial cells

Table I: Designation and source of R. salmoninarum isolates

Strain	Location of isolation / fish species source
1. Lea-1-74 (ATCC 33209)	Leaburg Hatchery, McKenzie River, OR Yearling Chinook Salmon
2. 33739 (ATCC)	Brook trout 81-10B-E-BK (NCMB 2196) D.L. Mitchum
3. Little Goose	Little Goose Dam, Idaho, Spring chinook salmon
4. D6	Oregon, USA, from Coho salmon held in Salt water
5. K-70	England, Rainbow trout
6. K-28	France, from the eye of pen reared Coho salmon
7. Kvilan	Kvilan, Iceland, Atlantic salmon
8. Grindsinc	Grindsinc, Iceland) Atlantic salmon
9. 684	Norway
10. K50	Norway, net pen cultured Atlantic salmon

were centrifuged at 6000 x g for 30 minutes (4°C) and the supernatant fluid saved for soluble antigen extraction.

Soluble antigen extraction.

Culture supernatants were filtered, as described above. The retentates, or high molecular weight fractions, were concentrated by 50% saturated ammonium sulfate (SAS) precipitation. After addition of the ammonium sulfate, the solutions were stirred for 3-4 hours at 4°C. The precipitate was removed by centrifugation at 6000 x g for 15 minutes (4°C and suspended in 10-20 ml of 10 phosphate buffered saline, pH 7.2 (PBS). The solution was reprecipitated twice, and the resuspended precipitate was dialysed extensively against PBS at 4°C. The dialysate was assayed for its protein by the method of Lowry et al. (1951).

Vibrio anguillarum extract.

The Vibrio extract was prepared from V. anguillarum strain LS-174 which had been formalin killed and stored frozen. Fifty mls thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for two hours. Cells were washed three times in 2% saline, centrifuged at 10,000 x g for 10 min at 4°C, resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed two times in acetone, centrifuging at 3,000 x g for 10 min and the pellet was dried overnight at 37°C. The pellet was ground to a fine powder with mortar and pestle and stored at 4°C. The soluble Vibrio extract used for these studies was prepared by boiling the powder in PBS at 10 mg/ml in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove particulates and filter sterilized. Protein concentrations were determined by the method of Lowry et al, (1951).

Nonoclonal antibodies.

Mice were immunized with 0.1 ml of a mixture of 1 O.D. (500 nm) formalin killed *R. salmoninarum* and 50 ug of soluble protein (ATCC 33209) in Freund's complete adjuvant (Difco, Detroit, MI). After 4 weeks mice were boosted with 25 ug of soluble protein mixed with 10 ug of *E. coli* LPS serotype 026:B6, TCA extract (Difco). Three days later a fusion with myeloma SP2 cells was performed and wells containing antibody secreting hybridomas (4D3 and 2G5) were cloned twice by limiting dilution and injected into mice for preparation of ascitic fluid. Ascites was harvested after 8-12 days, SAS precipitated, and resuspended in 10 mM PBS, diluted 1:1 with glycerol, and stored at -20°C . Both monoclonal antibodies were of the IgG_1 isotype as determined by the ICN Immunobiologics (Lisle, IL) isotyping kit.

Antibody (polyclonal) preparation.

Female New Zealand white rabbits were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) with a 1:1 emulsion of immunogen and Freund's complete adjuvant (FCA). The rabbits were rested 30 days then bled weekly. Seven weeks post-immunization the rabbits were boosted using the same protocol. The serum was aliquoted and stored at -70°C .

Immunogens used were the dialysed protein extracts from UF-KDM-2 (1.5 mg/ml) and KDM-2 (2.2 mg/ml).

Antisera from coho salmon were produced by monthly injections of 0.1 ml of a 1:1 mixture of soluble protein in FCA. Serum was aliquoted and stored at -70°C until used.

Biotinylation of antibody

Nonoclonal or polyclonal antibodies were dialyzed in 0.1 M NaHCO_3 and

reacted with a optimal ratio of 0.1 M solution of BNHS (Biotin-X Hydroxysuccinimide, Calbiochem CA) dissolved in distilled dimethyl formamide as described by Kendall et al. (1983). The mixture was reacted for one hour and dialyzed against three changes of PBS during a 24 hour period. The biotinylated antibody was diluted 1:1 in glycerol and stored at -20°C .

Polyacrylamide gel electrophoresis (PAGE)

Bacterial cells were centrifuged at 6000 x g for 0.5 hr, 200 ul of the pellet was then washed three times with 1 ml of 10 mM PBS and resuspended in an equal weight to volume of PBS and frozen at -20°C . Two microliters of cells were resuspended in 48 ul of double distilled water and 50 ul of sample buffer consisting of 120 mM tris base, 4% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol, and 3 mM bromophenol blue. The samples were then immediately boiled and electrophoresed on a 12% SDS-Page gel (83 x 103 mm mini-slab gel, Idea Scientific, Corvallis OR) with a constant current of 20 mAmps for approximately 1-5 hours. The samples were loaded in a duplicate fashion so that one half could be subjected to protein staining and the other half used for western blotting.

Western blotting

The proteins were transferred from the gel to nitrocellulose paper by electrophoresis at 60 V for 3.5 hours at 4°C according to a modified method of Towbin et. al. (1979). The nitrocellulose was subsequently blocked for 1 hour at 37°C with 3% BSA diluted in pH 8.0, tris buffered saline (TBS). A 1/500 dilution of McAB 4D3 in TBS was used to probe the blots for 1 hour at room temperature. Excess antibody was washed off with three, ten minute rinses with 0.1% tween(20)-TBS (T-TBS). A 1/200 dilution of goat anti-mouse immunoglobulin (Hyclone Logan UT) conjugated to horseradish peroxidase was

applied for 1 hr and rinsed 3x as described above. Visualization of the antigen bands was achieved using a substrate solution consisting of 2 ml of 4-chloro-naphthol (Bio-Rad)(3 mg/ml in methanol), 10 ul of 30% H_2O_2 and 10 mls, of 10 mM PBS. The other half of the blot was stained for total protein with 10 mls of Aurodyne colloidal gold solution for 3-5 hours.

Epitope analysis utilizing 4D3 and 2G5 antibodies

One hundred ul of a 5 ug/ml solution of either monoclonal, polyclonal antibody, or soluble protein (in carbonate-bicarbonate coating buffer, pH 9.6) was coated overnight at 17°C (Voller et al. 1976) onto EIA flatbottom plates (Costar, Cambridge, MA). The plates were inverted, with shaking, to remove excess protein. All wells were subsequently blocked for 1 hour with 150 ul of 1% BSA (Fraction V, Sigma) diluted in T-TBS. After washing three times with T-TBS and TBS, one hundred ul of a 5 ug/ml solution of soluble protein diluted in T-TBS or T-TX alone (negative control) were added to appropriate wells for one hour. After rinsing, 100 ul of 5 ug/ml biotinylated antibody was added for 1.5 hours. A 1/250 dilution of streptavidin-horseradish peroxidase (S-HRPO, Sigma) was added for 45 minutes. After the last rinse, 100 ul of substrate solution was added and color development was monitored spectrophotometrically at 405 nm on an EIA autoreader (Model 310, Biotek Instruments, Burlington, VT, USA). The substrate solution was a mixture of 10 mls of citrate buffer (pH 4.0), 10 ul of H_2O_2 and 75 ul of a 10 mg/ml solution of 2,2'-Azinobis 3-ethyl benzathiazoline sulfonic acid (ARTS) in distilled water.

Indirect fluorescent antibody techniques

Kidney smears of fish infected with the D6 isolate were made on pre-cleaned micro slides (VWR, CA) and fixed for 15 seconds with a 1:1

acetone:xylene solution. McAb 4D3 or 265 ascites was diluted to 1.5 ug/ml in 10 mM PBS, and 100 ul of **the** solution was added to the slide for 30 minutes. After a 5 minute wash with PHS, 50 ul of biotinylated goat anti-mouse immunoglobulin (Hyclone) diluted 1/100 in PBS was added for 15 minutes and washed for 5 minutes. A 1% methylene green solution was used as a counter stain. Slides were observed under a standard microscope (Zeiss) utilizing an IV, FI epi-fluorescent condenser and a 12 V,100 watt halogen tungsten light source. All ten of the R. salmoninarum isolates and three gram positive control bacteria were also analyzed by indirect FAT. Bacterial colonies from 15 day plate cultures were picked and air-dried on 3% gelatin coated slides which were subsequently heat fixed. Fluorescent staining was performed as described above.

ELISA for the detection of antibody to KDB soluble antigen

Briefly, the soluble protein antigens were diluted to a concentration of 5 ug/ml in carbonate buffer and coated on the wells of an ELISA plate. After an overnight incubation at 17°C the plate was washed and a 100 ul aliquot of hybridoma supernatant was incubated in the wells for one hr at room temperature. Following this incubation the plates were washed, and 100 ul of a 1/500 dilution of rabbit anti-mouse IgG-HRPO (Hyclone, Logan, Utah) was incubated on the plate for two hours at room temperature. After this incubation, the plates were washed and 100 ul of the substrate solution was added and read spectrophotometrically at 405 nm.

Bacterial agglutination assay

On a 96 well plate, 50 ul of PBS was placed in each well. The antiserum and normal serum from coho were added to the first two columns of the wells. A serial two fold dilutions of **the** sera was made by mixing the

contents of wells in the second column and transferring 50 ul to the next well and so on. The 50 ul from the last well was discarded. A heat killed RDB suspension ($1.0 \text{ O.D.}_{520\text{nm}}$) in PBS of 50 ul was added to each well from the second column onwards. The first column wells serve as buffer controls. The plate was allowed to incubate at room temperature for two hours and checked for any agglutination. If no reaction, the plate was then transferred to a 17°C incubator and checked every six hours. The antibody titer was represented as the highest dilution of serum which caused the bacterial agglutination.

Conjugation with tannic acid

Formalin-fixed *Vibrio* cells (0.5 ml packed cells) were washed three times with PBS (pH 7.2). PBS (pH 7.2) was prepared by mixing 100 ml of saline with 100 ml of phosphate buffer consisting of 24 ml of 0.15 M KH_2PO_4 and 76 ml of 0.15 M Na_2HPO_4 . The cells were then diluted with 7 ml of PBS (pH 7.2). Two ml of this *Vibrio* suspension was added to a 2 ml of tannic acid (0.05 mg/ml saline) solution. This suspension was mixed for 10 minutes in a 37°C water bath. The tanned cells were then washed three times in PBS (pH 7.2). One ml of soluble antigen (1 me/ml), 2 ml of the above suspension and 4 ml of PBS (pH 6.4) were mixed for 10 minutes at room temperature. PBS (pH 6.4) was prepared by mixing 100 ml of saline with 100 ml of phosphate buffer consisting of 32.2 ml of 0.15 M Na_2HPO_4 and 67.8 ml of 0.15 M KH_2PO_4 . To block the remaining protein binding sites on the *Vibrio* cells, the conjugated cells were incubated for 10 minutes in a gelatin solution (2 mg/ml saline). The conjugate was washed three times in saline and resuspended in saline to 2 ml.

Conjugation with glutaraldehyde

This form of conjugation basically follows the procedure of Avrameus

(1969). Heat killed KDB (Kidney Disease Bacterium) cells were washed with phosphate buffered saline (PBS, pH 7.2) and resuspended in PBS to give a 1.0 O.D. 520 nm suspension. Three ml of this suspension was pelleted at 6000 x g and three ml of Vibrio antigen extract (300 ug/sl protein) was added to the pellet and mixed thoroughly. Glutaraldehyde (25%) (Sigma, St. Louis, MO) (20 ul/ml of mixture) was added to the mixture and incubated for one hour at room temperature with occasional mixing. This preparation was centrifuged at 6000 x g for 30 minutes and washed with PBS twice and resuspended in PBS to 3 ml.

Chemiluminescence

A luminol stock solution was made by dissolving 1.77 mg of luminol in 1 ml of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). The stock solution was diluted 1/100 in PBS (pH 7.2) prior to use. A stock solution of phorbol myristate acetate (PMA) was also made in PBS at 2 mg/ml concentration and a working dilution of 1/200 made in PBS. Single cell suspensions of anterior kidney lymphocytes were prepared as described above. A measuring cuvette received 500 ul of luminol and 250 ul of PBS, followed by the addition of a 50 ul volume of the stimulant (PMA, KDSA, Vibrio cells or KDB cells). A suspension (2×10^7 cells/ml in RPMI) of 100 ul anterior kidney cells was added and the luminescence response was recorded on a potentiometric recorder (LKB instruments, Inc., Gaithersburg, MD).

Purification of Coho Immunoglobulins

Saturated ammonium sulfate (SAS) was used to precipitate the immunoglobulins (Igs) from the serum. A 50% SAS precipitation was performed twice on the serum, centrifuging at 1500 x g to pellet the precipitate each time. After the final spin, the pellet was resuspended in PBS and dialysed extensively against PBS at 4°C with at least two changes of the buffer. The

dialysate was filter sterilized and the protein content was **estimated by the** method of lowry et. al. (1951).

Affinity chromatography

Affinity chromatography of anti-KD immunoglobulins was based on the booklet "Affinity Chromatography" (Pharmacia, 1979) and Zoller et al. (1970). Briefly, 1 gm of CNBr activated Sepharose-4B (Pharmacia) was suspended in 50 ml of 1 mM HCl for 15 minutes. The suspension was washed and allowed to reswell on a sintered glass filter in 200 ml of 1 mM HCl. The KDSA was dissolved in coupling buffer [NaHCO_3 (0.1 M), pH 8.3) containing NaCl (0.5 M)]. The beads were then washed with coupling buffer and immediately transferred to the KDSA solution and mixed (a gel to buffer ratio of 1:2 was used). The mixture was mixed end-over-end on a multipurpose rotator (Scientific Industries Inc., Bohemia, N.Y.) at 4°C, overnight. The remaining active groups were blocked using **0.2 M glycine** in 0.5 M NaCl overnight at 4°C on the mixer. The gel was again washed with coupling buffer, followed by acetate buffer (0.1 M, pH 4.0) **containing** NaCl (0.5M) followed by a coupling buffer wash. The beads were packed into a column (10 cm x 9.7 cm) and the SAS purified immunoglobulins from coho-anti-RD serum were loaded into the column and incubated overnight at 4°C. The column was kept at 4°C throughout the chromatographic operation.

The column was then washed with PBS (pH 7.2, 0.01 M) and one ml fractions were collected (flow rate 30 ml/hour) till there was no detectable **O.D.**₂₈₀ nm (25 fractions) (Spectronic 21, Bausch & Lomb). This was followed by washing with high molar salt solution (2 M NaCl) and the fractions were collected and treated as above. The desorption of the anti-KD-antibody was accomplished by passing a 3 M potassium thiocyanate (KSCN) solution through the column and the fractions were collected and the O.D. measured. The fractions

that gave significant O.D. were dialysed against PBS at 4°C overnight with two changes of the buffer. SDS-PAGE, ELISA and protein estimation were performed on these fractions.

ELISA on the column_fractions

The method is similar to that described above. Briefly, 100 ul of KDSA (5 ug/ml) was coated on the wells of EIA plate and incubated overnight at 17°C. After blocking the wells with 1% BSA-TTBS for an hour the wells were washed with TTBS followed by TBS, three times each, Different dilutions of SAS purified coho-anti-KD serum in TTBS were incubated for 1 hr in the wells along with the dilutions of column fractions. The plate was washed as above, and an appropriate dilution of biotinylated Warr's monoclonal antibody, 1-14 (Kaattari and Yui, 1987) in TTBS was added and incubated for an hour at room temperature. After washing the plate, 1/100 dilution of streptavidin coupled to horseradish peroxidase (Sigma, St. Louis, MO) was added and incubated for 45 minutes. The wells were washed again and 100 ul of the substrate solution was added and the color development was measured spectrophotometrically at 405 nm as above.

Winn Assay - Neutralization with antiserum

Hyperimmune serum for the Winn assay was collected from seven coho salmon which had been immunized four times with 100 ug/fish of soluble protein over a 4 month period. Control sera was collected from two coho salmon. The hyperimmune sera contained 58.8 units of activity/ul of sera while the control sera had less than 0.08 units/ul as calculated by the ELISA titration method described below. One ml of each serum were mixed with 1 ml of 2.0 O.D. (500 nm) D6 KDB cells which had been washed and resuspended in PBS. After an incubation for 1.5 hrs., at 17 °C, the mixtures (0.1 ml/fish) were used to challenge 20 to 40 gm coho salmon (n=20). Mortalities were recorded daily.

Species Challenge with Renibacterium salmoninarum

Twenty-five coho salmon (Sandy Hatchery Stock), rainbow trout (Shasta strain) and chinook salmon (Abernathy Hatchery) were acclimated for two weeks in 30 liter indoor tanks and subsequently challenged intraperitoneally with 0.1 ml of 1 O.D. of the D6 KDB isolate. A students t-test was used to compare the mean time to death.

Comparison of anti-KDB produced in coho, rainbow trout, and chinook.

The D6 isolate was grown for 10 days in KDM-II supplemented with 10% calf serum. The cells were first treated with 0.3% formalin for 24 hours, and washed 2x in PBS. Subsequently, the cells were heat treated for 30 minutes at 56°C and stored at -20°C until use. Fifty rainbow trout, coho, and chinook salmon were immunized with 50 ul of 1.0 O.D (500 nm) cells emulsified 1:1 in FCA. Before immunization, and at 10 day intervals after immunization, sera from five fish from each species was collected and stored at -70°C until ELISA titration. Costar plate wells were coated overnight at 17°C with 10 ug/ml of D6 soluble protein diluted in coating buffer. The wells were subsequently blocked for 1 hour **with** 150 ul of 3% BSA T-TBS. After washing, 50 ul of dilutions of antiserum (pooled from five fish) or a hyperimmune standard, diluted in 3% BSA-TTBS, were added for 1.5 hours. During this step the plates were incubated at 17°C. To determine the amount of fish antibody present a 1/1000 dilution of the biotinylated monoclonal, 1-14, anti-fish antibody was added for one hour. Finally, a 1/200 dilution of S-HRPO was added for 0.5 hours. Substrate addition and optical density determination were as described previously. Calculation of the relative units of activity was as follows:

Relative units of activity =

ul of sera at 50% of hyperimmune standard
serum maximum reading

RESULTS AND DISCUSSION

Vaccine Trial

Thirty coho salmon were immunized by intraperitoneal injection of three distinct forms of immunogen. The first form consisted of heat-killed Renibacterium salmoninarum (HKDB) and Vibrio anguillarum extract (VAE) the second was comprised of the soluble antigen of KDR and formalin-fixed Vibrio anguillarum cells, and thirdly heat-killed KDB in Freund's complete adjuvant (FCA). As can be seen in figure 1, little protective effect was observed and very low antibody titers were induced (Fig. 2). It was felt that repeated immunizations or longer rest periods post inoculation may be required to elicit high antibody titers (see below). Also, although it is not apparent from these studies, some immunizations appear to lead to an earlier mean time to death (MTD) than is seen with control groups. Such exacerbation of the disease process has been noted when fish have been given a prior inoculation with KDB cell wall extracts (T. Evelyn, personal communication). This phenomenon, which is distinctly different from the increased MTD observed in previous studies (Raattari et al., 1987) suggests important considerations that must be addressed. This increased MTD may be due to either the possible toxigenicity of the KDB cells or extract used in the preparations of the immunogens, or alternatively, the injection of the extract is producing a state of hypersensitivity. Therefore, as the fish become immune, further exposure to the immunogen in the form of the live KDB challenge, produces a hypersensitivity reaction leading to granulomatous pathology resulting in earlier deaths.

If either of the two mechanisms stated above are responsible for the increased MTD, then it is essential that any toxigenic activities or determinants which may induce a hypersensitivity are neutralized. Conjugation procedures may play an important role in this putative neutralization. Our

VACCINE TRIAL

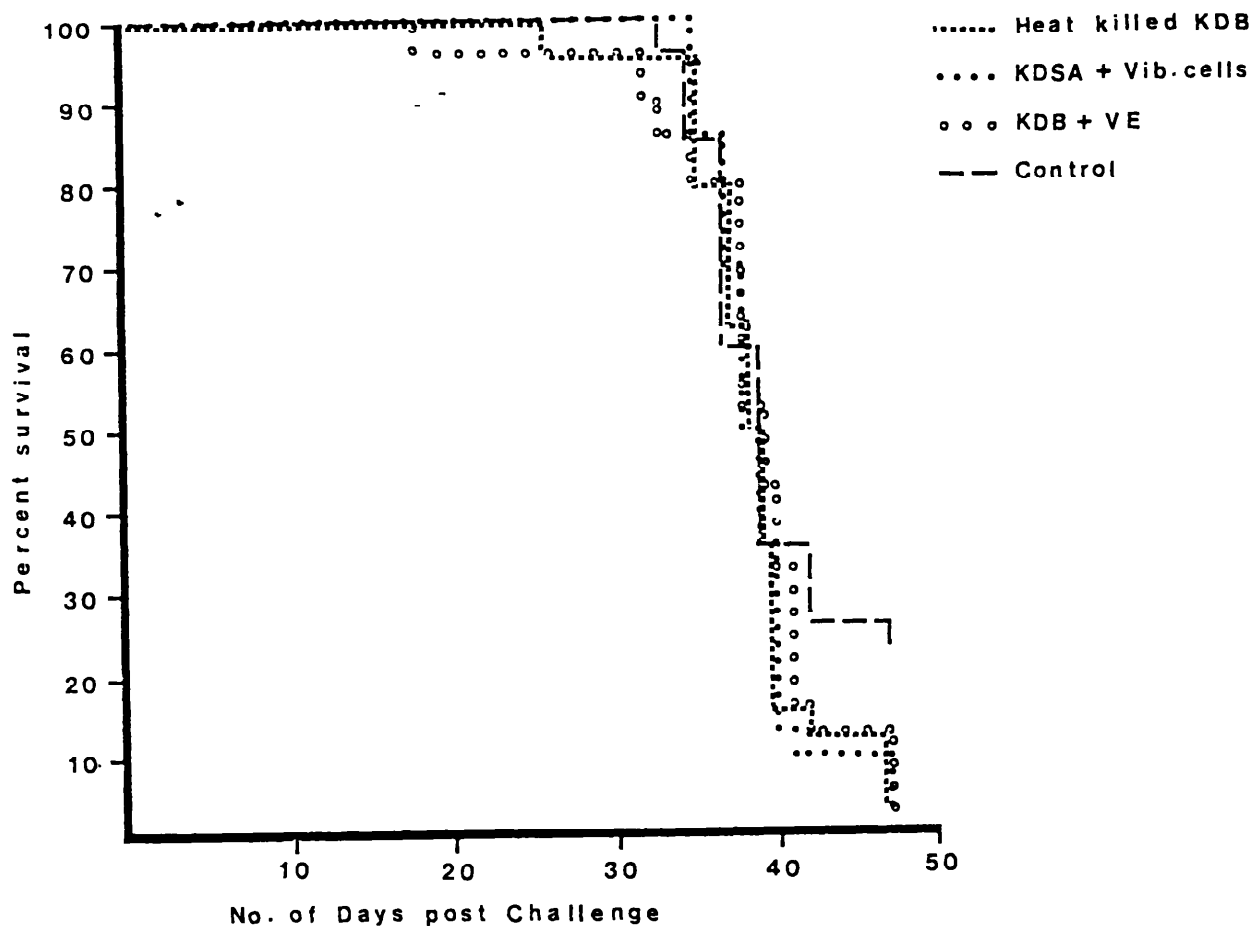


Figure 1: Challenge of prototype vaccine-immunized coho salmon with live *R. salmoninarum* by intraperitoneal injection (0.05 ml per fish of 1.0 O.D. 520 nm bacterial cell suspension). Groups of 30 fish were immunized (I.P.) with 0.05 ml of immunogens listed above.

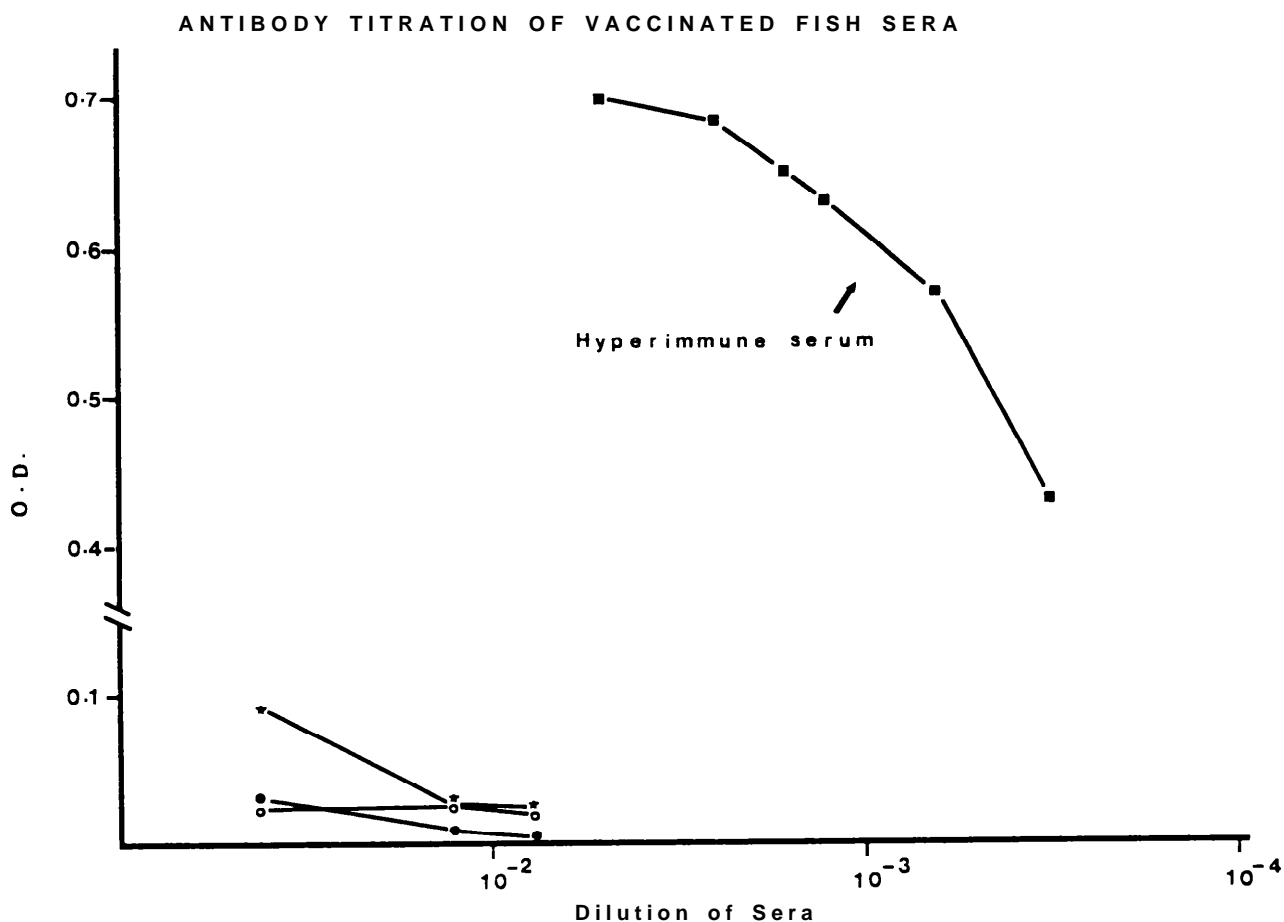


Figure 2: Serum antibody responses of prototype vaccine-immunised fish to soluble antigen. On day 21 post-immunization and prior to disease challenge three fish from each group were bled and serum antibody titers to kdsa were assessed by the elisa. The candidates were heat killed KDB plus vibrio extract (), formalin fixed Vibrio anguillarum plus KDSA (•) and heat killed KDB (*) in Freund's incomplete adjuvant (FCA). The amount of coho antibody present is proportional to the O.D. value.

previous studies (Kaattari et al., 1987) demonstrated that certain conjugation procedures can decrease the MTD, whereas others do not.

Serum Neutralization of Renibacterium salmoninarum

Questions as to the efficacy of serum antibodies in controlling bacterial kidney disease (BKD) have not, in the past, been rigorously addressed. Therefore, it was felt to be essential to determine the neutralizing capacity of salmonid antibodies. Further, determination of the titer of salmonid antibodies associated with protection could be used in the future in assessing the level of immunity induced by the various immunogens, as well as the appropriate time for challenge with live KDB after immunization. This study was accomplished by the use of what is termed a Winn assay. Figure 3 reveals that the immune serum (ELISA titer = 58 units of antibody activity /ul serum) provided significant protection as compared to PBS controls (<0.08 units/ill). It was of particular interest that the normal serum, which exhibited no demonstrable titer in the ELISA also appeared to protect the challenged fish to the same degree as the immune serum. This protection is reflected in the difference in the MTD: 37.522.8 days for immune serum, 37.722.7 days for normal serum, and 29.622.9 days for the controls (no serum). Values given are the mean times to death \pm the standard error of those means.

This protective capacity of the "normal" serum was unexpected. However, when this serum was tested for KDB bacterial agglutination titers it was found to possess a titer comparable to that of serum from specifically immunized fish (Table II). One possible reason for this difference could be that two different isotypes are responsible for the titers observed, one isotype may be detected by the ELISA only, while the other can only be observed in the bacterial agglutination assay. The mechanisms by which these two assays operate are considerably different. The ELISA employs an anti-trout

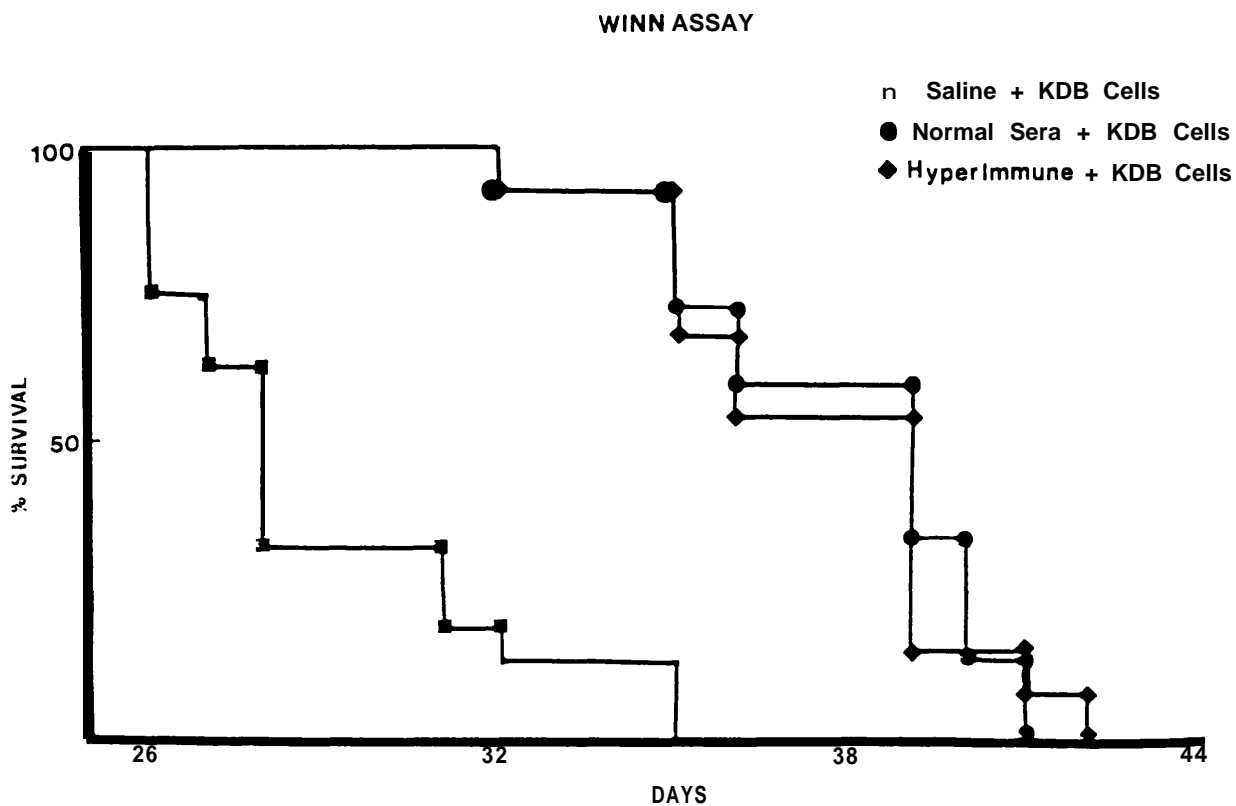


Figure 3. Winn assay for detection of neutralizing fish antibodies. Three groups of 20-40 gm coho salmon were challenged with 0.1 al of a 1:1 mixture of serum a 2.0 O.D. suspension of D6 cells diluted in either saline (n=16), normal coho sera (n=15 or coho hyperimmune anti-soluble protein sera (n=13). Mortalities were recorded daily.

Table II. Agglutination titers of coho sera from seven sources

COHO SERUM SOURCE	TITER
Source # 1	1 : 16
Source # 2	1 : 128
Source # 3	1 : 64
Source f 4	1 : 128
Source # 5	None
Source # 6	None
Source # 7	None

Source #4 is the hyperimmune serum and source #2 is the normal serum used in the Winn assay.

immunoglobulin (1-14) reagent (which also binds salmon immunoglobulin (Kaattari and Yui, 1987)) for the detection of salmonid antibody, whereas the agglutination assay depends solely on the presence of salmonid antibody. Therefore, if the 1-14 monoclonal antibody used in the ELISA can only detect certain isotype(s), an isotype which may have protective capabilities would not be detected by this ELISA.

Future experiments will be performed to determine whether agglutinating and/or ELISA titers are more indicative of a protective response. This may be essential in the analysis of the induction of protective immunity, since the production of the appropriate isotype(s) may be crucial to successful immunity.

Antibody titration of vaccinated fish sera

In a previous report (Kaattari et al., 1987) it was demonstrated that although some amount of increased survival was seen with certain conjugates, the elicited antibody titer was barely detectable. It was, therefore, deemed essential that higher levels of antibody be induced prior to challenge with KDB. In these studies 30 coho salmon were vaccinated with either a single or two intraperitoneal injections (separated by 40 days) of either one of four antigen preparations emulsified in Freund's complete adjuvant (FCA). They were; 1) soluble antigen (KDSA), 2) KDSA conjugated to formalin-fixed Vibrio anguillarum cells, 3) heat-killed KDB (HKDB) or 4) HKDB conjugated to VAE. Controls consisted of PBS emulsified in FCA or no injection. Titers were determined for fish from each group prior to challenge. In the previous study fish were screened for their antibody titers 21 days after immunization. Since no substantial titer was detected at that time (Fig 2), fish in this assay were tested at 60 days post immunization. As can be seen (Fig 4), all serum titers from antigen-immunized salmon were substantial. It is of

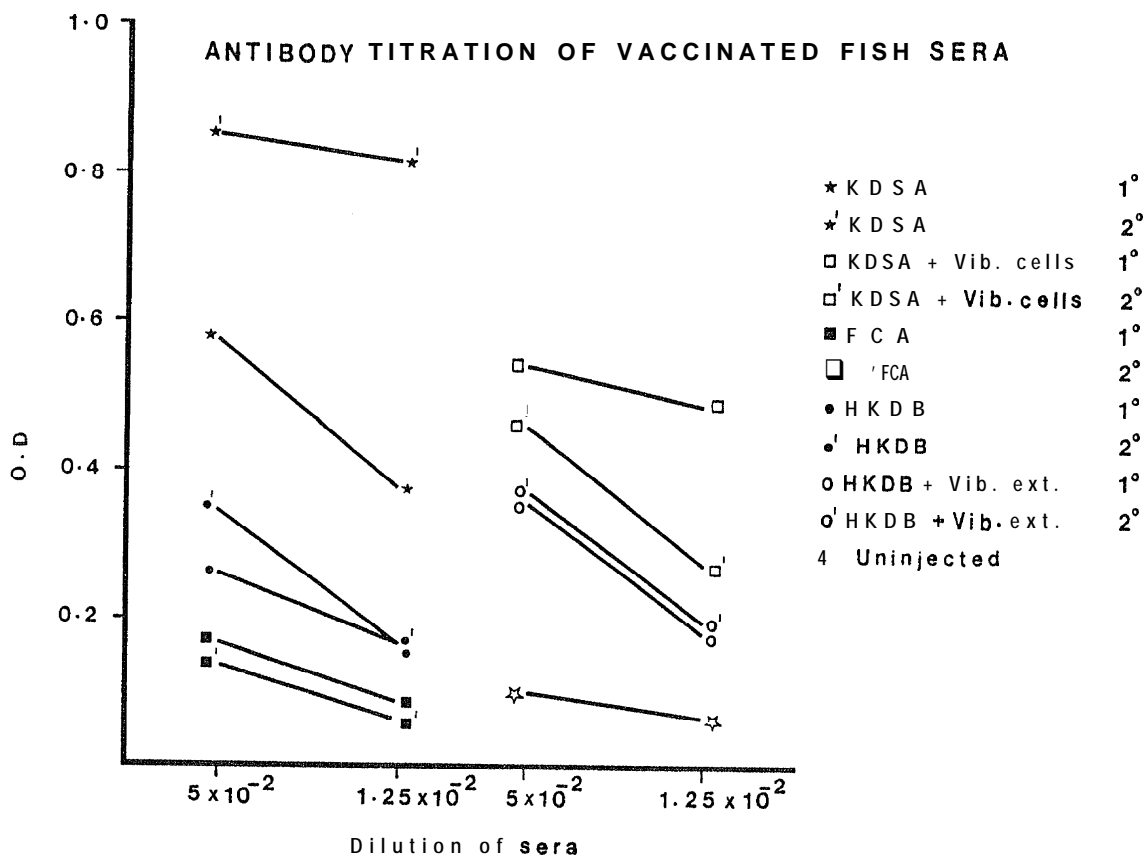


Figure 4: Serum antibody responses of vaccinated fish to soluble antigen, On day 60 post-immunization and prior to disease challenge, two fish per group were bled and serum antibody titers to KDSA were assessed by the ELLISA. The amount of coho antibody present is proportional to the O.D. value and each point represents the average of the two fish titers,

particular interest that the secondary response to KDSA produced a titer similar to that of the hyperimmune serum (O.D. maximum = 0.9). Heightened secondary antibody responses are usually expected for protein antigens in mammals. We have also recently reported that this form of immunological memory can be seen in rainbow trout using the protein antigen, keyhole limpet hemocyanin (Arkoosh and Kaattari, 1987). This now appears to be the case for coho salmon in response to KDSA. The use of "booster" injections has proven to be essential in generating immunity to a variety of human and animal toxins and pathogens. These fish are currently being challenged with live KDB to test the efficacy of this treatment.

Susceptibility of salmonids to Renibacterium salmoninarum challenge

Successful immunizations have only been reported in the literature in Salmo species (Paterson et al., 1981; McCarthy et al., 1984), but not in Oncorhynchus species. We, therefore, felt that it would be important to perform a standard challenge with similarly sized fish (coho = 19+6 gm, chinook = 29+4 gm, rainbow = 28+4 gm) to determine if there was any significant differences in the mortality rates between different salmonid species. The challenge (Fig 5) revealed that coho salmon were the least susceptible (MTD + 1 S.E.=31.4+2.4), followed by rainbow trout (28.3+5.1), and chinook salmon (25.222.2). The possible reasons for these differences in susceptibility may be due to some immune component. For example, it may be possible that coho salmon *or* species of the Salmo genus may be able to produce antibodies which have unique specificities. These antibodies may be essential in eliminating KDB more efficiently than possible with chinook salmon antibodies. Studies are currently underway to determine if such antibody specificities or isotypes exist (below).

Comparative antibody responses to Renibacterium salmoninarum among salmonids

KD CHALLENGE

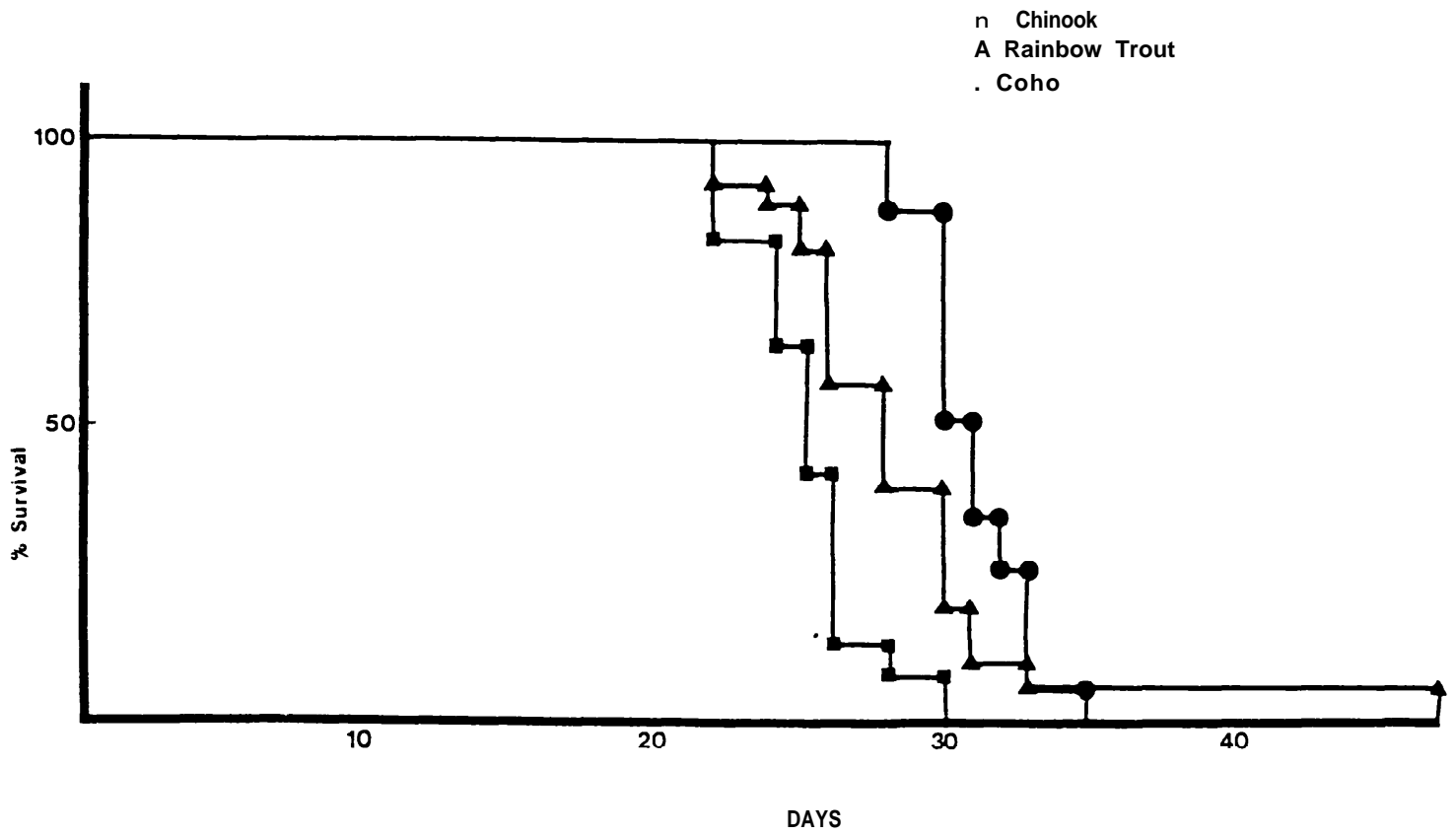


Figure 5. Twenty-five similarly sized coho, chinook, and rainbow trout were challenged with 0.1 ml of a 1 O.D. suspension of a ten day culture of r. salmoninarum (d6 isolate). Mortalities were recorded daily.

Groups of 50 **chinook**, trout, and coho salmon were immunized with **heat-killed KDB** and soluble antigen emulsified in Freund's complete adjuvant. The serum antibody titers induced by this immunization were determined by use of the ELISA at various days post inoculation. It can be seen from figure 6 that rainbow trout produce a much greater antibody response to KDB (47.6 units/ul) followed by coho salmon (10.0 units/ul), and then chinook salmon (4.7 units/ul). This experiment suggests that a greater antibody titer to KDB antigens may be induced in rainbow trout than in salmon, when the antigen is administered under comparable conditions. One possible flaw in this analysis, may be the use of 1-14 anti-trout immunoglobulin reagent in this assay. Previous work has suggested to us that this reagent appears to recognize immunoglobulins from these species equally well, however a stringent quantitative assay to determine the equivalency of these immunoglobulins is currently being conducted. Since trout immunoglobulin was the immunogen used in the preparation of this antibody, this assay may have a higher affinity for it. If not, it would appear that trout possess a greater ability to produce antibodies in response to KDB antigens.

Isolation of protective anti-KDB antibodies

The previous studies described above suggest that salmonids may be able to produce protective antibody responses, and these antibodies may be of a particular isotype or binding specificity. If this hypothesis is correct it would be essential to determine the isotype(s) of these antibodies, under what conditions are they induced, and whether salmonids of various species share these isotypes. Further, it would be important to determine the binding site specificities (idiotypes) that would be associated with a protective antibody **response**. These goals will be accomplished by the isolation of anti-isotypic and anti-idiotypic antibodies (monoclonal) which can identify these salmonid

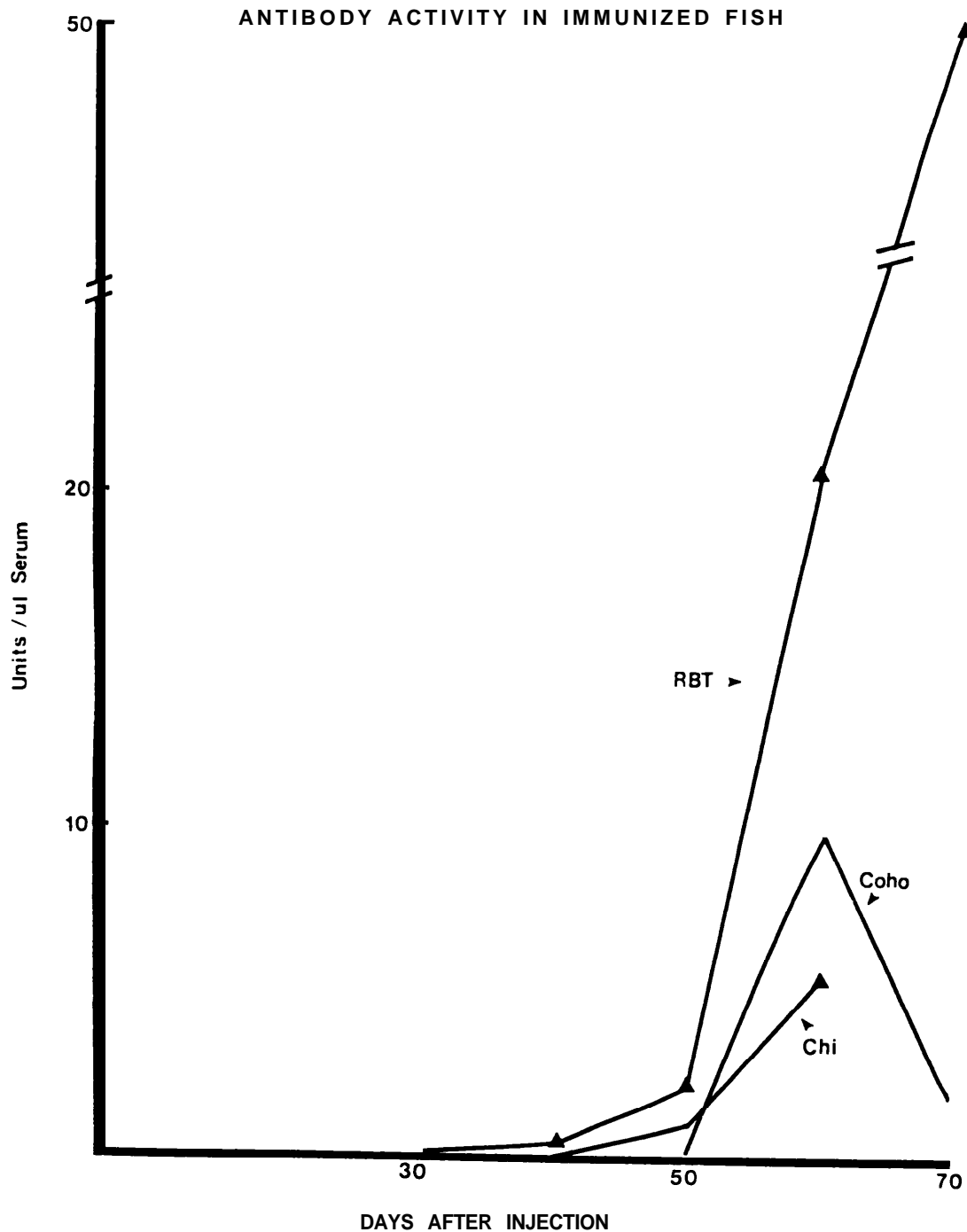


Figure 6. Fifty coho, chinook, and rainbow trout were each immunized with 50 ul of 1.0 OD (500 nm) of killed *R. salmoninarum* (D6 isolate) suspended 1:1 in FCA. Sera was taken from 5 sacrificed fish of each group at 10 day intervals. Equal amounts of sera were pooled and the specific anti-soluble protein antibodies were assayed by an ELISA. Units of antibody activity were calculated by comparison to a coho hyperimmune standard sera as described in the methods.

antibodies. This is being accomplished by the affinity purification of coho anti-KDB antibodies, and the immunization of mice with these specific antibodies. Thus far, affinity purification of these antibodies has been successful (Table III) antibodies of very high activity. These antibodies are being used in the immunization of BALB/c mice for the production of monoclonal antibodies.

Chemiluminescent activity - Comparison of two stocks of coho salmon

One aspect of host defenses that may be very important in the development of resistance to BKD is cellular immunity. It has been observed that macrophage or phagocytic activity may be very important in the progress of BKD (Young and Chapman, 1978). One particular measurement of this form of immunity is through the use of chemiluminescence. Measurement of chemiluminescent activity is a direct measure of the generation of oxidative molecular species which act to destroy pathogens. These molecules are released when a phagocytic cell responds to a stimulus, such as a bacterium or a synthetic compound, such as phorbol myristic acetate (PMA). In the first study depicted in figure 7, we have evidence that not all, apparently normal, coho salmon respond equally well to the stimulus of PMA. It is of interest that stock #2 has had no reported outbreaks of BKD, whereas stock X1 has had a history of outbreaks. Stock #2 (N>10) indices measure routinely around 170 mV, whereas stock #1 (N=8) routinely measures (2 mV. This may indicate that these fish have a chronic subclinical infection with KDB, which in turn may be depressing their phagocytic activity or, alternatively, this stock has a low level of chemiluminescent activity. This low activity, then in turn, may be responsible for an increased susceptibility to BKD. Future studies, will be designed to determine if certain stocks have characteristic levels of chemiluminescent activity, and if there is any relationship to BKD incidence.

Table II[: Characteristics of affinity purified Coho-anti-KD.

Antibody activity:	
1. Total activity in the SAS purified Coho-anti-RD that was loaded onto the column.	830 units/mg
2. Specific activity of the affinity purified antibody (of the KSCN elution).	5333 units/mg
Molecular weights of the bands of the purified fraction (SDS-PAGE).	70,000 68,000 doublet 60,000 27,000 23,000

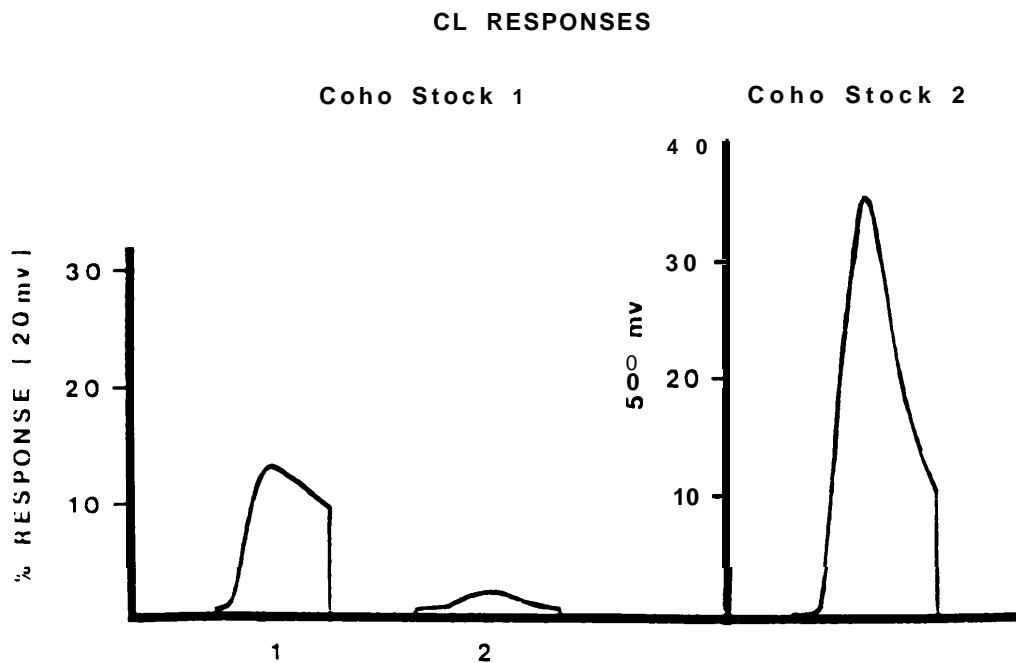


Figure 7. Chemiluminescent responses of anterior kidney lymphocytes of two stocks of Coho salmon to PMA. Two samples were tested from Coho stock #1. These responses are representative of 8 samples of stock #1 and >10 samples of stock #2

Chemiluminescent activity - Use in the evaluation of prototype vaccines

Determination of the potency and efficacy of the various vaccine candidates in eliciting a cellular immune response would be of great benefit. Armed with such capabilities we may be able to identify the most potent candidates for the induction of a cellular response prior to the massive immunization efforts required for *in vivo* trials. This could then represent immense savings in time and effort. The value of such an assay can be seen in the results depicted in figure 8. Heat-treated NKD cells conjugated to VAE induce substantially higher responses than soluble antigen alone, Vibrio anguillarum cells, HKDB cells or Vibrio cells conjugated to soluble antigen.

Chemiluminescent activity - Effect of soluble antigen

Previous studies (Kaattsri et al., 1987) have indicated that the soluble antigens elaborated by KDB may have toxic activity. This toxigenic activity appeared to induce decreased hematocrits and reduce antibody responses *in vitro*. The suppression of the antibody response to trinitrophenylated-lipopolysaccharide may be the result of a toxic effect upon the macrophage. This was felt to be a distinct possibility since this antibody response is highly dependent upon the adherent cell, which loses its adherence during co-culture with soluble antigen. Figure 9 is a representation of the chemiluminescent responses of leukocytes to PMA in the presence of soluble antigen and various controls. The response to PMA alone was approximately 200 mV, however upon co-incubation with soluble antigen the response dropped to 5mv, or 97.5% of the control. Co-incubation with equivalent amounts of bovine serum albumin or an equivalent amount of the medium from which the soluble antigen was isolated (KDN-2) demonstrated no suppression of the chemiluminescent response. This effect indicates that the

CL RESPONSE OF CONJUGATES

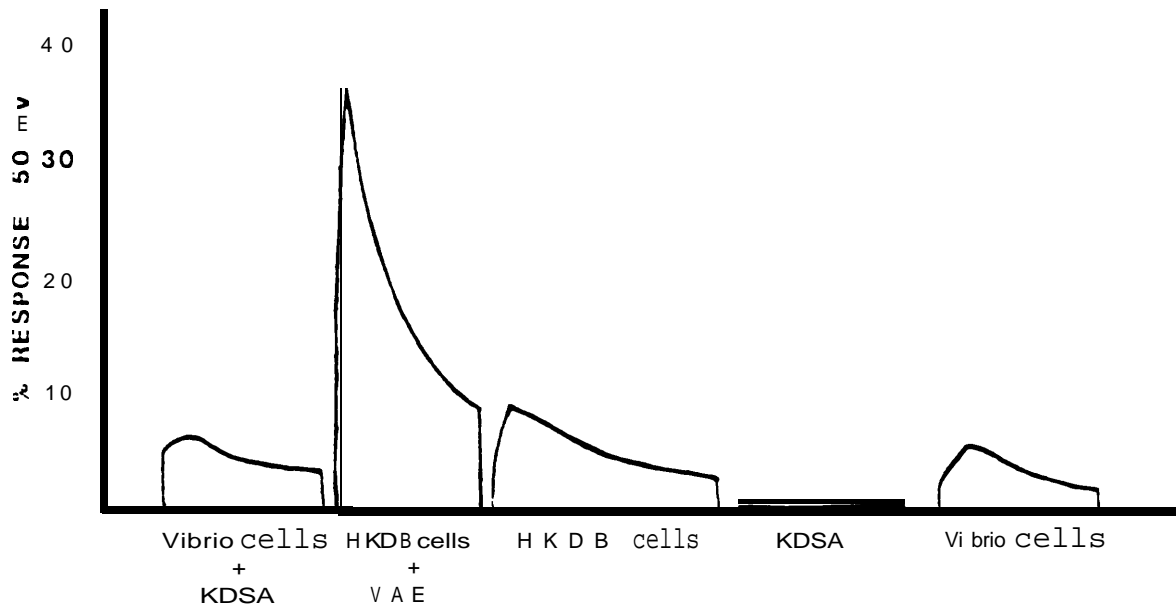


Figure 8: Chemiluminescent responses of **anterior** kidney (AK) lymphocytes of coho to the vaccine candidates: formalin killed vibrio cells plus KDSA, heat killed KDB cells plus vibrio extract and heat killed KDB cells. KDSA and formalin killed vibrio cells are controls. AK lymphocytes were coincubated with the above preparations for 45 min. at 17°C and mixed with luminol before measuring the response.

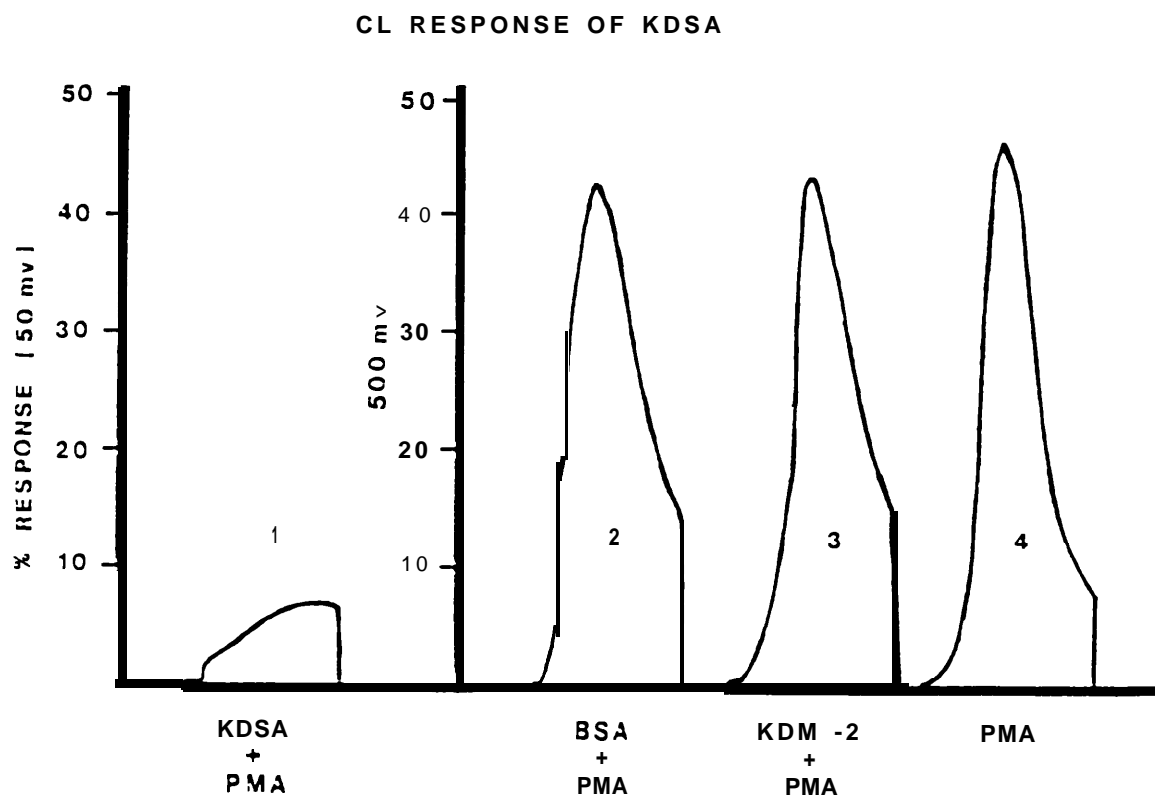


Figure 9: Chemiluminescence responses of anterior kidney lymphocytes. 1: Column purified fraction of KDSA plus PMA, sensitivity at 50 mV. 2: A control protein BSA plus PMA, 3: column fraction of KDM - 2, same as that of KDSA and quantitatively same, 4: PMA as control. 1,2 and 3 were incubated with AK lymphocytes for 45 min. at 17°C before measuring the response. The sensitivity for 2,3 and 4 is at 500 mV

soluble antigen, which we have demonstrated to be elaborated in great quantities in infected fish (Turaga et al., 1987) may have an extremely detrimental effect on one of the cell types essential to the production of an antibody response. It must also be realized that the use of the soluble antigen in its native form, for the purposes of vaccination may be ill-advised. Continuing studies on the preparation of prototype vaccine material, will concentrate on the effects of soluble antigen used as an immunogen and on generation of toxoids from this material.

Renibacterium salmoninarum strain characterization by western blotting

The cellular proteins of ten geographically distinct isolates (Table I) were electrophoresed and tested by western blotting with the monoclonal antibody (McAb) 4D3, which was described in the previous annual report (Kaattari, et al., 1987). The antibody reacted with one major protein (Mr 57 kd) and four minor proteins (Mr 58, 50, 45, 43 kd) present in all isolates (Fig 10.1, 11.1, 12.1). No cross-reactive proteins were detected in the gram positive control bacteria (Fig 13.1). Additionally, no cross-reactive proteins were detected in western blots of the gram negative salmonid pathogens Vibrio anguillarum or Aeromonas salmonicida (Fig 14.1). Protein profiles of the ten strains of bacteria also suggest that the strains are very homogeneous (Fig 10.2, 11.2, 12.2). The complex of antigens recognized by the two monoclonal antibodies correlates with a 57 kd surface protein named antigen F by Getchell et al. (1985). Turaga et al. (1987) have also observed that this is the predominant protein found in experimentally infected fish sera. The electrophoretic profiles of Aurodye-stained nitrocellulose blots of the 10 isolates appear homogeneous, supporting the observed uniformity in other characteristics such as biochemical properties, and cell wall carbohydrates (Bruno and Munro, 1986, Fiedler and Draxl, 1986).

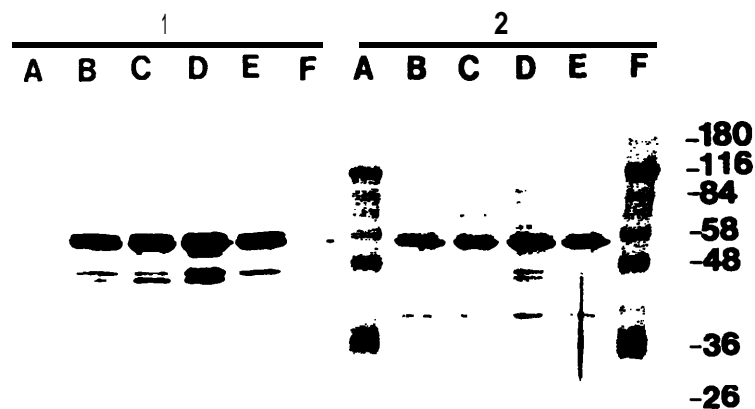


Figure 10. Western Blot analysis of cellular antigens of *Renibacterium salmoninarum* isolates recognized by monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining (panel 2). Lanes are as follows; A and F) Pre-stained molecular weight markers (Sigma), B) ATCC 33209, C) ATCC 33739, D) C D6 isolate, E) Little Goose isolate.

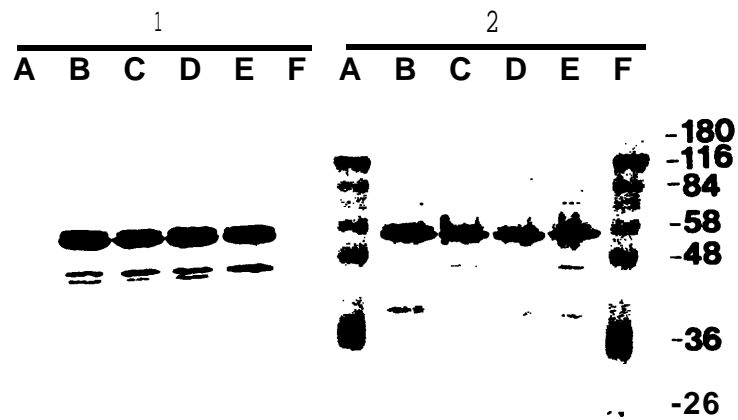


Figure 11. Western blot analysis of cellular antigens of *Renibacterium salmoninarum* isolates recognized by monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining (panel 2). Lanes are as follows; A and F) Pre-stained molecular weight markers, B) ATCC 33209, C) K-28 isolate, D) K-70 isolate, E) 684 isolate.

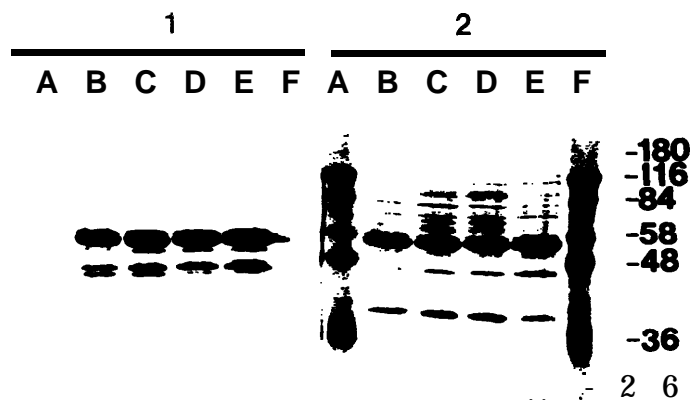


Figure 12. Western blot analysis of cellular antigens of Renibacterium salmoninarum isolates recognized by monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining (panel 2). Lanes are as follows; A and F) Pre-stained molecular weight markers, B) ATCC 33209, C) K-50 isolate, D) Grindsinc isolate, E) Klivan isolate.



Figure 13. Western Blot analysis of cellular antigens of Renibacterium salmoninarum and gram positive control bacteria recognized by monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining-(panel 2). Lanes are as follows; A and F) Pre-stained molecular weight markers, B) ATCC 33209, C) Lactobacillus piscicola, D) Streptococcus facium, E) Streptococcus fecalis.

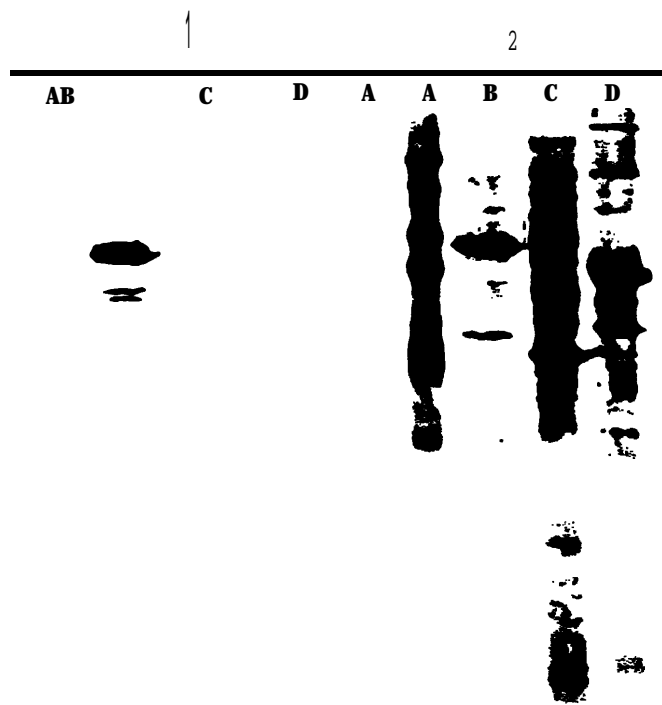


Figure 14. Western blot analysis of cellular antigens of Renibacterium salmoninarum and gram negative control bacteria recognized by monoclonal antibody 4D3 (panel 1), and total protein profile as detected by colloidal gold staining (panel 2). Lanes are as follows; A) Pre-stained molecular weight markers, b) ATCC 33209, C) Vibrio anguillarum, D) Aeromonas salmonicida.

Arakawa et al. (1987) have produced monoclonal antibodies to a heat stable antigen and found antigenic diversity among isolates. They speculate that the antibodies might be to antigen F epitopes which are isolate specific.

ELISA epitope analysis

Recognition of distinct epitopes by the two monoclonal antibodies was ascertained by the **use** of an antibody capture ELISA. In this assay antibody was bound to the wells, soluble antigen added, and **the** same antibody (biotinylated) was added to determine if any epitopes were still available (Figure 15). In this situation, no increase in optical density was observed compared to background, when either 4D3 or 2G5 were coated onto the plate. This indicated that all. antigens were bound by the single epitope recognized by the first, or capture antibody. Thus, all antigen molecules are in the identical orientation. Use of the heterologous monoclonal. or polyclonal antibody as the second antibody demonstrated a high level of binding (80 - 100% of the maximal O.D), indicating that most or all of the other epitopes were still available. As a positive control, to demonstrate that the biotinylated antibodies were equally functional, all biotinylated antihodies produce the same amount of activity when incubated with wells directly coated with soluble antigen. This analysis **suggests that** each monoclonal antibody recognizes a single epitope. In addition, the two recognized epitopes are different. Antigenic variability of epitopes on one protein is not uncommon; Buchanan et al. (1987) characterized 14 separate epitopes on a 65 kd Mycobacterium leprae protein. Thirteen epitopes were common to other Mycobacterial species while one McAb recognized an epitope which was species-specific. Monoclonal antibodies 4D3 and 2G5, however recognize two separate epitopes which are common to all tested isolates of KDB, yet are species specific.

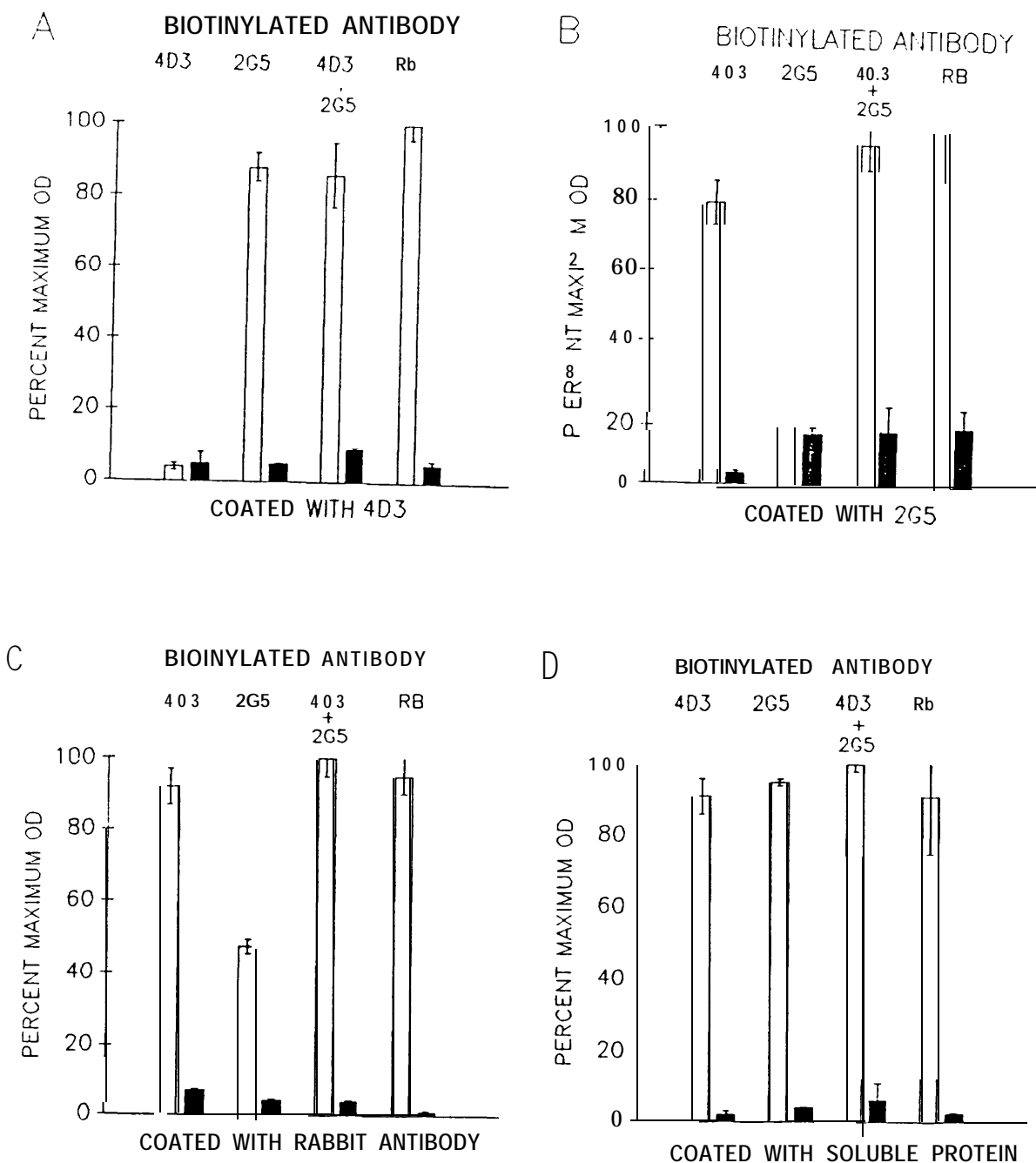


Figure 15. Epitope analysis by indirect ELISA. Triplicate wells were coated with either monoclonal antibody 4D3 (panel A), 2G5 (panel B), polyvalent rabbit antisera (panel C), or R. salmoninarum soluble proteins (panel D) used as a positive control. Either T-TBS or soluble protein was then added, incubated and washed off. Biotinylated antibody (4D3, 2G5, or polyvalent) were used to assay apparent soluble antigen in each well. Data is expressed as a percentage of the maximum OD (panel A=.753, B=.397, C=.751, D=.811). Darkened columns represent background levels in which no soluble antigen was added,

Indirect fluorescent antibody analysis

An indirect fluorescent antibody assay was used to detect unique surface epitopes of the KDB cells. Experimentally infected chinook salmon kidney smears of the ten isolates tested positive (Fig 16), while none of the other gram positive control bacteria stained positive.

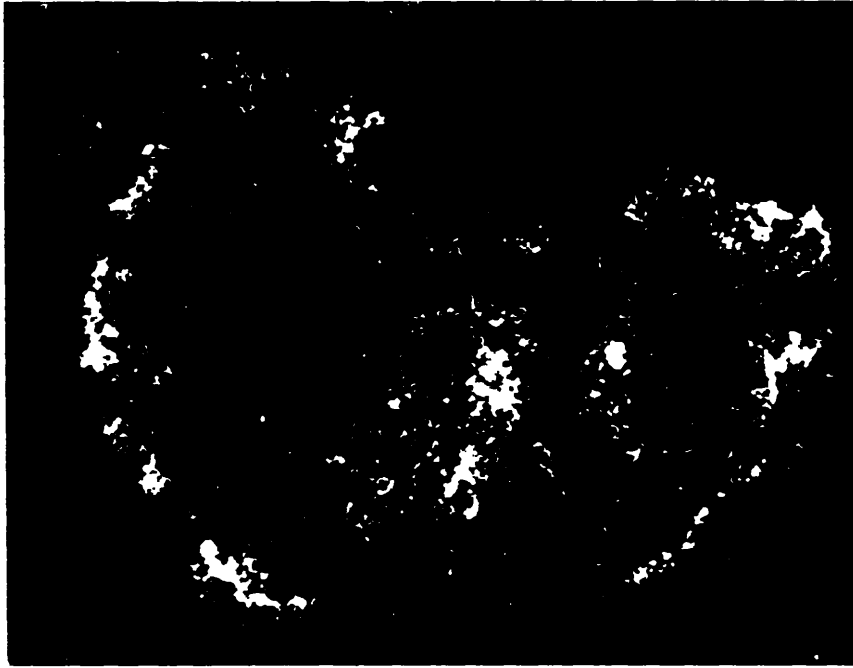


Figure 16. Indirect fluorescent antibody stain of the anterior kidney of a chinook salmon which had been experimentally infected for 20 days with the D6 isolate of R. salmoninarum. Kidney tissue was fixed on the slide and fixed with 1:1 xylene-acetone solution and probed with 1.5 ug/ml of monoclonal antibody 4D3 ascites as described in the methods section.

SUMMARY AND CONCLUSIONS

The data presented here demonstrates the following major conclusions:

- 1) Sensitive western blot techniques demonstrate that two monoclonal antibodies recognize two different epitopes of the soluble antigen molecules.
- 2) The monoclonal antibodies recognize common antigens in isolates of KDB found globally. Further, they possess no cross-reactivity to various gram positive and gram negative fish pathogens.
- 3) Protective immunity to BKD may require "booster" injections of vaccine material and/or a minimum of two months in order to produce a suitably high titer which could protect salmon.
- 4) There may be species-specific differences in the ability to resist BKD and in the relative ability to produce antibodies to the organism.
- 5) chemiluminescence can be used to assess the state of cellular immunity and/or phagocytic activity in KDB immunized fish.

These studies indicate that induction of a state of protective immunity will most likely require long term immunization schedules (2-3

months) and that this immunity may be highly dependent on the **genetic** ability of the salmon to produce the correct antibodies. We feel that all prototype vaccines should be monitored closely as to the level of antibody and chemiluminescent activity required for a state of immunity. Current studies are directed at devising the optimal regimens for the induction of such immunity.

SUMMARY OF EXPENDITURES

1.	Salaries (including personnel and benefits)	\$ 45,606.35
2.	Travel and transportation (including per diem)	4551.54
3.	Nonexpendable equipment and material (greater than \$1000 per item)	20,987.55
4.	Expendable equipment and material (sensitive in nature)	34,797.76
5.	Operations and maintenance (including computer services and publications)	0.00
6.	Overhead	28,361.80
7.	The currently approved budget	135,242.00
8.	Current budget period	7/1/87-6/31/88
9.	Cumulative expenses to date	134,304.65

REFERENCES

- Arakawa, C., Sanders, J., and Fryer, J. 1987. Production of monoclonal antibodies against Renibacterium salmoninarum. J. Fish Dis. 10:249
- Arkoosh, M. R., and Kaattari, S. L. 1987. The effect of early aflatoxin B₁ exposure on in vivo and in vitro antibody responses in rainbow trout, Salmo gairdneri. J. Fish Biology 31:19
- Avrameus, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Immunochem. 6:24
- Banner, C.R., Rohovec, J.S. and Fryer, J.L. 1983. Renibacterium salmoninarum as a cause of mortality among chinook salmon in salt water. J. World Maricult. Soc. 14:236.
- Belding, D.L. and Merrill, B. 1935. A preliminary report upon a hatchery disease of the salmonidae. Trans. Am. Fish Soc. 65:76.
- Billingham, R.E., Brent, L. and Medawar, P.B. 1953. Actively acquired tolerance of foreign cells. Nature 172:603.
- Bruno, D. and Munro, A. 1986. Uniformity in the biochemical properties of Renibacterium salmoninarum isolates obtained from several sources. FEMS Micro. Lett. 33:247
- Buchanan T., Nomaguchi, H., Anderson, D., Young, R., Gillis, T., Britton, W., Ivanyi, J., Kolk, A., Clos, O., Bloom, B., and Mehra, V. 1987. Characterization of antibody-reactive epitopes on the 65-kilodalton protein of Mycobacterium leprae. Infect. and Imm. 55:1000
- Bullock, G.L., Stockey, H.M. and Wolf, K. 1975. Bacterial kidney disease of salmonid fishes. Fish and Wildlife Service, Fish Diseases Leaflet No. 41. U.S. Department of the Interior, Washington, D.C.

- Earp, B.J., Ellis, C.H., Ordal, E..J. 1953. Kidney disease in young salmon. Spec. Report Ser.No. 1, State of Washington, Dep. Fish. 72p.
- Evealyn, T.P.T., Hoskins, G.E., and Bell, G.R. 1973. First record of Bacterial Kidney Disease in an apparently wild salmonid population in British Columbia. Jour. Fish. Res. Board Can. 30:1578.
- Evelyn, T.P.T., Ketcheson, J.E. and Prosperi-Porta, L. 1984. Further evidence for the presence of Renibacterium salmoninarum in salmon eggs and for the failure of povidane-iodine to reduce the intra-ovum infection rate in water-hardened eggs. J. of Fish Diseases 7~173.
- Fiedler, F., and Drax; R. 1986. Biochemical and immunochemical properties of the cell surface of Renibacterium salmoninarum. J. Bact. 168:799
- Frantsi, T., Flwelling, T.C. and Tdswell, K.G. 1975. Investigations on corynebacterial kidney disease and Diplostomulum sp. (eye -fluke) at Margaree Hatchery, 1972-1973. Tech. Rep. Ser. No. Mar/T-75-a Res. Development Branch, Fish and Marine Serv., Dep. Environ. Halifax, Nova Scotia, Canada, 30p.
- Fryer, J.L. and Sanders, J.E. 1981. Bacterial Kidney Disease of salmonid fish. Ann. Rev. Microbiol 35:273
- Getchell, R.G., Rohovec, J.R., and Fryer, J.L. 1985. Comparison of Renibacterium salmoninarum isolates by antigenic analysis. Fish Pathol. 20:149
- Hendricks, J.D. and Leek, S.L. 1975. Kidney disease postorbital lesions in spring chinook salmon (Onchorhynchus tshawytscha). Trans Am. Fish Soc. 104:805.
- Hunn, J.B. 1964. Some patho-physiologic effects of bacterial

- kidney disease in brook trout. Proc. Soc. Exp. Biol. Med. 117:383.
- Kaattari, S.L. and Yui, M. 1987. Polyclonal activation of salmonid B lymphocytes. Dev. Comp. Immunol. 11:155.
- Kaattari, S., Holland, N., Turaga, P., and Wiens, G. 1987. Development of a vaccine for bacterial kidney disease. Annual Report, Bonneville Power Administration. Portland, OR. 71p.
- Kendall, C., Ionescu-Matiu, I., and Dressman, R. G. 1983. Utilization of the Biotin/Avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). J. Imm. Methods 56:329
- Rimura, T. and Awakura, T. 1977. Bacterial Kidney Disease of salmonids. First observation in Japan. Bull. Jpn. Soc. Sci. Fish 48:143.
- Lowry, O.H., Rosebrough, N.J., Farr, D.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 205-276.
- MacRaness, G.B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vitro. J. Exp. Med. 129:973.
- McCarthy, D., Croy, T., and Amend, D. 1984. Immunization of rainbow trout, Salmo gairdneri Richardson, against bacterial kidney disease: preliminary efficacy evaluation. J. Fish Dis. 7:65.
- Ordal, E.J. and Earp, B.J. 1956, Cultivation and transmission of etiological agent of kidney disease in salmonid fishes. Proc. Soc. Exp. Biol. Med. 92:85.
- Paterson, W.D., Desautels, D. and Weber, J.M. 1981. The immune response of Atlantic salmon, Salmo salar L., to the causative agent of bacterial kidney disease, Renibacterium salmoninarum.

- Jour. of Fish Diseases 4:99.
- Pharmacia booklet. 1979. Affinity chromatography. Principles and Methods.
Pharmacia Fine Chemicals AB. Uppsala, Sweden.
- Sanders, J.E. and Fryer, J.L. 1980. Renibacterium salmoninarum
gen. nov., sp. nov., the causative agent of bacterial kidney
disease in salmonid fishes. Int. Jour. of Syst. Bacteriol.
30:496.
- Smith, I.W. 1964. The occurrence and pathology of Dee disease.
Fresh water and salmon. Fish. Res. 34:3
- Snieszko, S.F. and Griffin, P.J. 1955. Kidney disease in brook
trout and its treatment. Prog. Fish-Cult. 17(1) : 3.
- Towbin, H. et al. 1979. Electrophoretic transfer of proteins from polyacryl-
amide gels to nitrocellulose sheets. Procedure and some applications.
Proc. Natl. Acad. Sci. 76:4350
- Turaga, P., Wiens, G., and Kaattsri, S. 1987. Bacterial kidney disease:
The potential role of soluble protein antigen(s). J. Fish Biol.
31:191
- Voller, A., Bidwell, D. E., and Bentlett, A. 1976. Enzyme immunology in
diagnostic medicine (Theory and Practice). Bull. World Health Organ.
53:55
- Wedemeyer, G.A. and Ross, A.J. 1973. Nutritional factors in the
biochemical pathology of corynebacterial kidney disease in the
coho salmon (*Oncorhynchus kisutch*). Jour. Fish. Res. Board Can.
30:296.
- Wolf, J.W. and Dunbar, C.E. 1959. Methods of infecting trout with
kidney disease and some effects of temperatures on experimental
infections. U.S.D.I. Fish and Wildl. Serv. Spec. Sci. Rep.
Fish. No. 286. 8p.

- Wood, J.W. and Wallis, J. 1955. Kidney disease in adult chinook salmon and its transmission by feeding to young chinook salmon. Fish Comm. of Oregon Res. Briefs 6:32.
- Young, C. L. and Chapman, G. B. 1978. Ultrastructural aspects of the causative agent and renal histopathology of bacterial kidney disease in brook trout (Salvelinus fontinalis). J. Fish. Res. Bd Can. 35:1234
- Zoller, M., and Matzka, S. 1976. Antigen and antibody purification by immunoadsorption: elimination of non-biospecifically bound proteins. J. Immunol. Methods 11:287.